ORIGINAL RESEARCH

Exosomes Derived from Human Palatal Mesenchymal Cells Mediate Intercellular Communication During Palatal Fusion by Promoting Oral Epithelial Cell Migration

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Purpose: Exosomes are important "messengers" in cell-cell interactions, but their potential effects on palatal fusion are still unknown. This study aimed to explore the role and mechanism of exosomes derived from palatal mesenchymal cells in epithelial-mesenchymal communication during palatogenesis.

Methods: The expression of exosome marker CD63 and CD81 in palatal cells during palatogenesis was detected by immunofluorescence staining. After being purified from the supernatant of human embryonic palatal mesenchymal (HEPM) cells, exosomes (HEPM-EXO) were characterized by nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM) and Western blot. HEPM-EXO were co-cultured with human immortalized oral epithelial cells (HIOEC). The effects of HEPM-EXO on the cell proliferation, migration, apoptosis and epithelial-mesenchymal transition (EMT) of HIOEC were evaluated. The proteins encapsulated in HEPM-EXO were analyzed by proteomic analysis.

Results: The extensive expression of CD63 and CD81 in palatal epithelial and mesenchymal cells were continuously detected during E12.5~E14.5, suggesting that exosomes were involved in the process of palatal fusion. The expression of CD63 was also observed in the acellular basement membrane between the palatal epithelium and the mesenchyme in vivo, and HEPM-EXO could be internalized by HIOEC in vitro, suggesting that exosomes are potent to diffuse through the cellular tissue boundary to mediate palatal cell-cell communication. Exposure of HEPM-EXO to HIOEC substantially inhibited the proliferation and stimulated the migration of HIOEC, but had no significant effect on cell apoptosis and EMT. Proteomic analysis revealed the basic characteristics of the proteins in HEPM-EXO and that exosomal THBS1 may potentially regulate the cell behaviors of HIOEC, which needs further verification. Gene ontology (GO) analysis uncovered that the proteins highly expressed in HEPM-EXO are closely related to wound healing, implying a promising therapeutic opportunity of HEPM-EXO in tissue injury treatment with future studies.

Conclusion: HEPM-EXO mediated cell-cell communication by regulating cell proliferation and migration of oral epithelial cells during palatogenesis.

Keywords: epithelial-mesenchymal interactions, extracellular vesicles, palatogenesis, proteomics, wound healing

Introduction

The mammalian palate is a vital organ which separates the oral and nasal cavities. The palate forms from shelves of mesenchyme covered by epithelium, wherein mesenchymal cells are derived from cranial neural crest cells of ectoderm, and epithelial cells are derived from pharyngeal ectoderm.[1] The palatogenesis undergoes palatal shelf outgrowth, elevation above the tongue, horizontal growth toward the midline and, ultimately, fusion with one another

to form the intact roof of the mouth. After the palatal shelves meet, the palatal medial edge epithelial (MEE) cells are removed to achieve mesenchymal confluence.[2] The removal of palatal MEE cells is critical and essential for palatal fusion, which would otherwise result in cleft palate,[3] one of the most common birth defects.[4] In addition to signals from the epithelium itself, mesenchymal signals also can regulate epithelial cell behaviors that contributes to MEE degeneration. For instance, the deficiency of palatal mesenchymal Smo function results in compromised palatal epithelial cell proliferation.[5] In addition, mesenchymal Msx1 regulates the expression of BMP4, which regulates the epithelial Shh expression.[6] Epithelial-mesenchymal interaction is active and intricate during palatal fusion. Nevertheless, our understanding of how the adjacent mesenchymal cell signals are transmitted to epithelial cells is remarkably limited.

In the last two decades, the intercellular communication via secreted extracellular vesicles has been solidly established.[7] As a subtype of extracellular vesicles, the nano-sized vesicles, exosomes (30~150 nm), are actively secreted by the majority of cells. They are enclosed by a lipid bilayer and carry a variety of biomolecules, including proteins, nucleic acids, lipids, and metabolites. When exosomes are taken up by other cells, these cargoes are transferred and affect the behaviors of recipient cells.[8] Exosome-mediated intercellular communication is involved in multiple organogenesis, including craniofacial ones.[9] During tooth formation, exosomes secreted by dental epithelial and mesenchymal cells reciprocally evoked cell differentiation and matrix synthesis.[10] Exosomal miRNAs transported from salivary mesenchyme epigenetically regulates genes controlling salivary epithelial cell proliferation.[11] Therefore, we hypothesized that exosome-mediated cell-cell communication may also play a part in the process of palatal fusion. The present study aimed to evaluate the effects of palatal mesenchymal cell-derived exosomes on epithelial cell behavior and to uncover the possible mechanism underlying exosome-mediated cell-cell communication by proteomic analysis.

Materials and Methods
Animals
All animal experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals at Wuhan University and approved by the Medical Ethics Committee of the School of Stomatology at Wuhan University (Protocol number S07919080E). Wild-type C57BL/6 mice were housed under specific-pathogen-free conditions. Mice were mated for 12 h, and the day of the vaginal plug is considered E0.5. Embryos were obtained from timed pregnant mice.

Histology
Embryonic heads were harvested at E12.5~E14.5 and fixed in 4% paraformaldehyde (PFA) at 4°C overnight. Samples were dehydrated in serial concentrations of ethanol, embedded in paraffin, and serially sectioned at 5 μm using a microtome (RM2255; Leica Biosystems Inc., USA).

Immunofluorescence Staining
Tissue sections were deparaffinized and boiled in EDTA Antigen Retrieval Solution (Beyotime Biotechnology, China) for 20 min in a microwave. After blocking in Quick Block (Beyotime Biotechnology) buffer for 15 min at room temperature, slices were incubated with primary antibodies at 4°C overnight. Slides were washed three times in phosphate buffered saline (PBS) before incubation with secondary antibodies for 1 h at room temperature. The following antibodies were used in this study: anti-rabbit CD63 (1:200, ab217345, Abcam, UK), anti-rabbit CD81 (1:100, A5270, Abclonal, China), anti-mouse P63 (1:100, ab735, Abcam), Anti-rabbit IgG (H+L) F(ab')2 Fragment (Alexa Fluor 488 Conjugate, 1:400, 4412, Cell Signaling Technology/CST, USA), Anti-rabbit IgG (H+L) F(ab')2 Fragment (Alexa Fluor 555 Conjugate, 1:400, 4413, CST), Anti-mouse IgG (H+L) F(ab')2 Fragment (Alexa Fluor 555 Conjugate, 1:400, 4409, CST). Nuclei were counterstained with 4′,6- diamidino-2-phenylindole dihydrochloride (DAPI). Cells on coverslips were observed and photographed using a fluorescence microscope (Leica Biosystems Inc., USA).

Cell Cultures

The human embryonic palatal mesenchymal (HEPM) cell line was purchased from American Type Culture Collection (USA), and cultured in Minimum Essential Medium/Earle's Balanced Salts (MEM/EBSS) (HyClone, USA) supplemented with 10% foetal bovine serum (FBS) (Gibco, USA) and 1% penicillin/streptomycin (PS). Human immortalized oral epithelial cells (HIOEC) were kindly provided by Professor Huan Liu from School & Hospital of Stomatology, Wuhan University (Hubei, China), and cultured in KGM Gold Basal Medium (Lonza, Switzerland) supplemented with KGM Gold SingleQuots (Lonza), 2% FBS and 1%PS. Cells were cultured in a humidified incubator at 37°C in an atmosphere of 5% CO_2.

Exosome Isolation, Quantification, and Characterization

Exosomes were purified from cell culture supernatant of HEPM cells cultured for 48h in MEM supplemented with 10% exosome-depleted FBS (Biological Industries, Israel). Supernatants were centrifuged at 2000 g for 30 min at 4°C to remove dead cells and debris, followed by filtration with 0.22 μm pore filters (Merck Millipore, USA) to remove small debris and larger vesicles. Supernatants were then concentrated using Amicon Ultra-15 Centrifugal Filter Device (100kDa MWCO, Merck Millipore). Exosomes were precipitated using Total Exosome Isolation (TEI) (Invitrogen, USA) reagent according to manufacturer's instructions. Briefly, 1 mL of the concentrated supernatants was mixed with 0.5 mL of TEI solution and incubated at 4°C overnight. Samples were spun down for 1 h at 10,000 g and 4°C. The pellet was resuspended in 100 μL of PBS and stored at −80°C before use. For exosome quantification, exosome proteins were extracted using radioimmunoprecipitation assay (RIPA) buffer and the protein concentration was determined using the BCA Protein Assay Kit (Pierce Biotechnology, USA). To examine exosome size distribution and particle concentration, nanoparticle tracking analysis (NTA) was performed using a ZetaView instrument (Particle Metrix, Germany). Exosome morphology was observed using transmission electron microscopy (TEM) (Hitachi, Japan) as previously described.[12]

Exosome Labeling and Uptake

To observe the exosomal uptake by HIOECs, HEPM cell-secreted exosomes (HEPM-EXO) were labeled with BODIPY TR ceramide fluorescence dye (Invitrogen). Briefly, 10 μM of the dye was added to 100 μL of the exosomes and the mixture was incubated at 37°C for 20 min (protect from light). Excess unincorporated dye was removed with Exosome Spin Columns (MW3000, Invitrogen) according to the manufacturer's instruction. The fluorescently labeled exosomes were added to HIOECs cultured in 24-well plate and incubated at 37°C for 2 h. Cells were fixed with 4% PFA at room temperature for 20 min. Nuclei were counterstained with DAPI. Samples were imaged with a fluorescence microscope.

Western Blot

Total proteins of HEPM-EXO, HEPM and HIOEC lysates were extracted as described above. Proteins were loaded and separated by SDS-PAGE gel, transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Germany) and immunoblotted using primary antibodies against GM130 (1:1000, ab52649, Abcam), ALIX (1:1000, ab186429, Abcam), β-ACTIN (1:5000, AC028, Abclonal), CD81 (1:1000, A5270, Abclonal), E-CADHERIN (1:1000, ab231303, Abcam), VIMENTIN (1:2000, A19607, Abclonal), THBS1 (1:500, A2125, Abclonal) and POSTN (1:1000, A9009, Abclonal). Membranes were incubated with HRP Goat Anti-Rabbit IgG (H+L) (1:5000, AS014, Abclonal) at room temperature for 1 h, visualized by WesternBright ECL Western Blotting HRP Substrate (K-12045-D50, Advansta, USA) and imaged by an Odyssey CLx Imaging System (LI-COR, USA).

Cell Counting Kit-8 (CCK-8) Assay

HIOECs at logarithmic phase were seeded in 96-well plates. After cells attached to the wall, exosomes (40 or 400 ng/μL) or PBS (control group) were added to HIOECs respectively and 10 μL of the CCK-8 (Dojindo Laboratories, Japan) reagent were added to each well and incubated at 37°C for 1 h at 0, 24, 48, 72, 96, 120 and 168 h. The absorbance was measured using a microplate reader (Synergy LX, BioTek, USA) at a wavelength of 450 nm.

5-Ethynyl-2'-Deoxyuridine (EdU) Incorporation Assay

EdU incorporation assay was performed using the BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 555 (Beyotime Biotechnology) according to manufacturer's instruction. After exosome stimulation, HIOECs were incubated with 10 μM EdU reagent for 2 h and fixed with 4% PFA at room temperature for 15 min. Following incubation with Click reaction mixture containing Click Reaction Buffer, $CuSO_4$, Azide 555 and Click Additive Solution for 30 min at room temperature (protect from light), cells were washed with PBS for three times. Nuclei were counterstained with DAPI. Samples were imaged with a fluorescence microscope.

TUNEL Assay

For TUNEL analysis, the One Step TUNEL Apoptosis Assay Kit (Beyotime Biotechnology) was used according to manufacturers' instruction. After exosome stimulation, HIOECs were fixed with 4% PFA for 30 min, permeabilized with 0.3% Triton X-100 (in PBS) for 5 min and immersed in TUNEL reaction mixture at 37°C for 1 h in the dark. Nuclei were counterstained with DAPI. Samples were imaged with a fluorescence microscope.

Scratch Assay

At 90~95% confluence, HIOECs were scratched using a sterile 200 μL pipette tip, washed with PBS and incubated with exosomes (40 or 400 ng/μL) or PBS (control group) in serum-free medium for 24h. Images of scratched areas were captured at 0, 18 and 24 h time points. The percentage of cell migration was measured by ImageJ software (NIH).

Transwell Assay

HIOECs were seeded onto the upper chamber of the Transwell (Corning, USA) and cultured in serum-free medium. Exosomes (40 or 400 ng/μL) or PBS (control group) were added to the lower chamber. Cells were incubated at 37°C for 16 h. Thereafter, the non-migrated cells in the upper chamber were removed using cotton swabs. Cells that migrated below the Transwell layer were fixed with 4% PFA for 15 min and stained with 0.1% crystal violet (G1014, Servicebio, China) at room temperature. Cells were imaged with a light microscope (Leica) and stained areas were quantified using ImageJ software.

Four-Dimensional (4D) Label-Free Quantitative Proteomics

Proteins of HEPM-EXO and HEPM cells were extracted as described above, followed by 4D label-free quantitative proteomic analysis (Genechem, China). Mass spectral data were analyzed using MaxQuant software version 1.6.17.0. The cutoff of global false discovery rate (FDR) for peptide and protein identification was set to 0.01.

Statistical Analysis

All experiments were repeated at least 3 times. Statistical analyses were conducted using GraphPad Prism 9 (GraphPad Software Inc., CA). One-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test was applied to analyze the differences among groups. The results are presented as the mean ± standard deviation (SD), and $p < 0.05$ was considered statistically significant. Functional protein association network was analyzed using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (http://string-db.org).

Results

Exosomes Present in Palatal Mesenchymal and Epithelial Cells During Palatal Fusion

To explore the potential effects of exosomes on palatogenesis, we first examined whether exosomes exist in palatal mesenchymal and epithelial cells during palatal fusion. We detected the expression level of widely recognized exosome markers, CD63 and CD81,[13] by immunofluorescence staining. The basal cell layer of palatal epithelium was indicated by P63 staining. The results showed that, from the initiation of palatogenesis (E12.5) to palatal shelf elevation (E13.5) and fusion (E14.5), CD63 (Figure 1A) and CD81 (Figure 1B) were continuously expressed in the palatal mesenchymal and epithelial cells. Importantly, CD63-positive exosomes were also detected in the acellular basement membrane at E13.5 (Figure 1C), indicating that exosomes spread unidirectionally or reciprocally across the basement membrane.
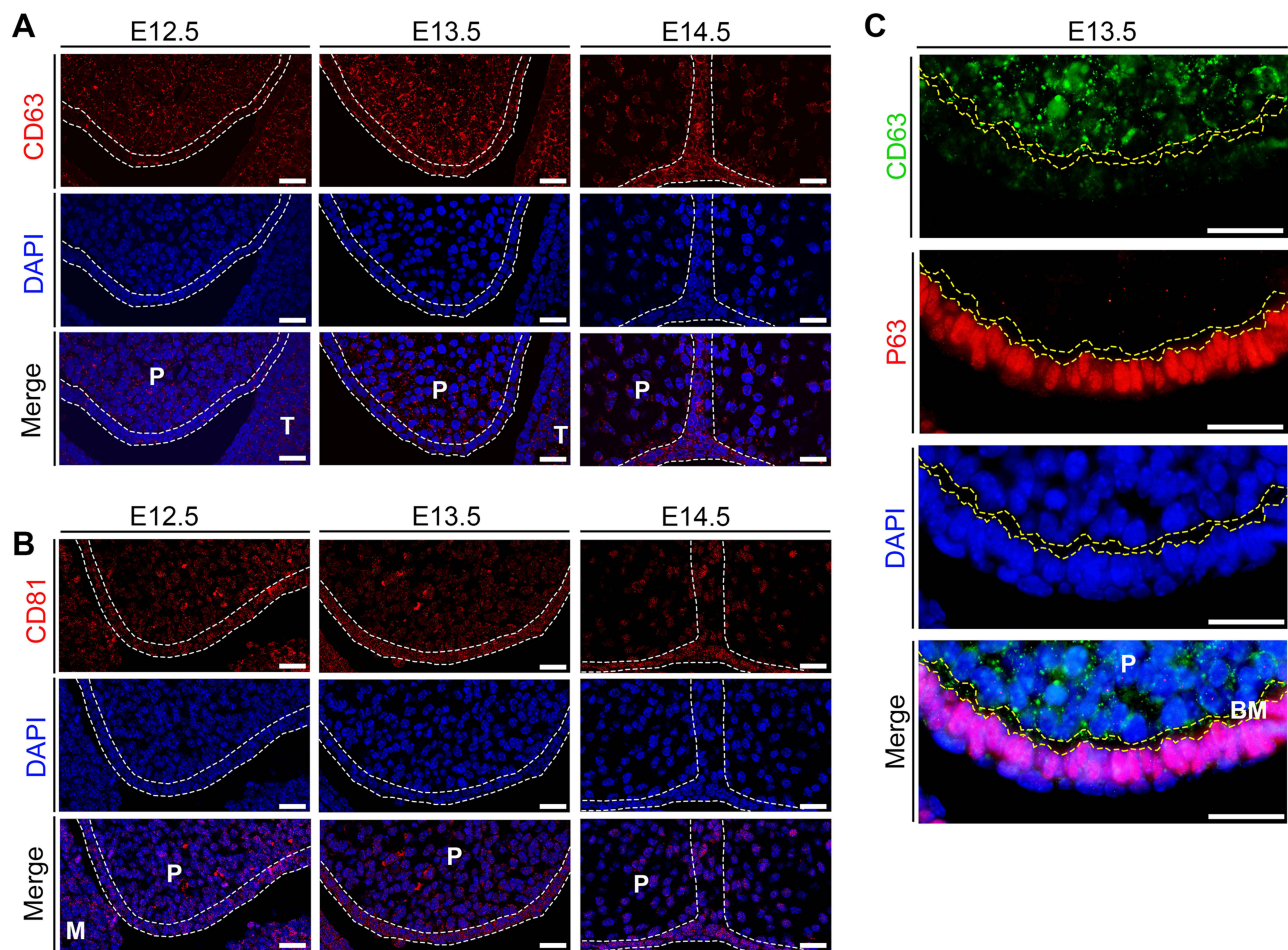
Figure 1 The presence of exosomes in palatal cells and basement membrane during embryonic palate development. (**A**) Immunofluorescence staining of CD63 (red) and DAPI (blue) from E12.5 to E14.5. Dotted white lines indicate the epithelial compartment of palate. (**B**) Immunofluorescence staining of CD81 (red) and DAPI (blue) from E12.5 to E14.5. Dotted white lines indicate the epithelial compartment of palate. (**C**) Immunofluorescence staining of CD63 (green), P63 (red) and DAPI (blue) at E13.5. Dotted yellow lines indicate the acellular basement membrane. Bar=20 μm.
Abbreviations: P, palate; M, mandibular; T, tongue; BM, basement membrane.

Exosomes Derived from Palatal Mesenchymal Cells Can Be Internalized by Oral Epithelial Cells

Since exosomes can diffuse through the basement membrane and might be internalized by recipient cells in vivo, we further utilized in vitro methods to testify whether mesenchymal exosomes can be transferred to epithelial cells. Exosomes were extracted from HEPM cell supernatant and characterized by NTA, TEM and Western blot. The NTA results (Figure 2A) showed that a large proportion of these vesicles were approximately 55 to 130 nm in diameter (mean = 80.7 nm), which is within the characteristic diameter range of 30 to 150 nm of exosomes. To verify that these isolated vesicles were not non-membranous particles of a similar size, their morphology was observed using TEM. The image (Figure 2B) showed a typical bowl-like structure with membranes and ovoid shapes. Western blot results showed that these exosomes were not only ALIX, β-ACTIN and CD81 positive but also GM130 negative, suggesting the absence of Golgi or cell contamination (Figure 2C). Thereafter, exosomes were labeled with a fluorescence dye and co-cultured with HIOEC to visualize the process of exosome endocytosis. Results (Figure 2D) showed a clear uptake of labeled exosomes by HIOEC, while the PBS control group only exhibited limited background staining, suggesting sufficient washing to remove excess unbound dye. These results indicated that palatal mesenchymal cell-secreted exosomes can be endocytosed by HIOEC.
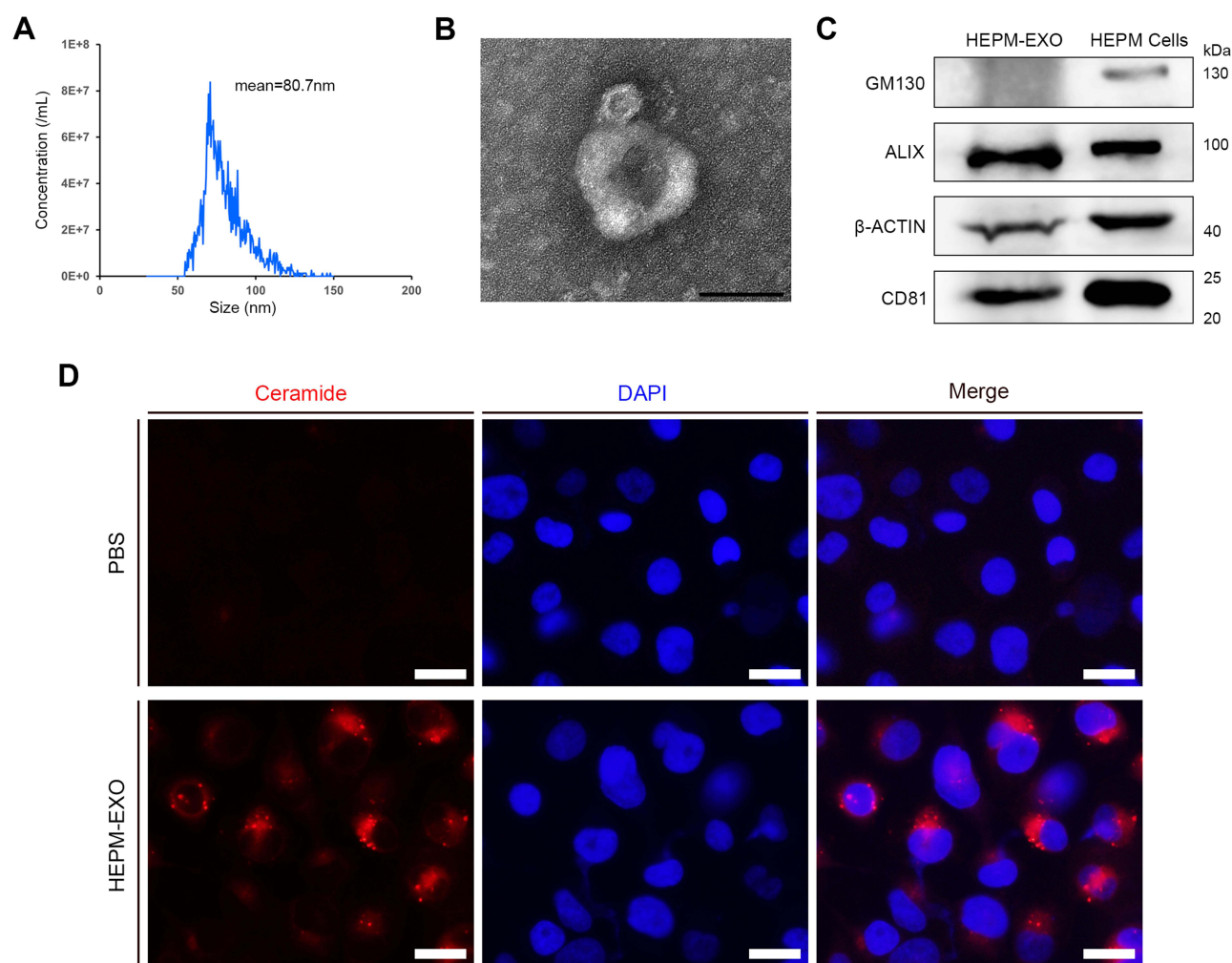
Figure 2 HEPM-EXO were internalized by HIOEC in vitro. (**A**) Nanoparticle tracking analysis (NTA) showed that the diameter range of exosomes derived from HEPM cells (HEPM-EXO) was 55 to 130 nm (mean=80.7 nm). (**B**) The image captured by transmission electron microscopy (TEM) showed a typical bowl-like structure with membranes and ovoid shapes. Bar=100 nm. (**C**) Western blot analysis showed positive expression of exosome markers (ALIX, β-ACTIN and CD81) and negative expression of GM130 in HEPM-EXO proteins. (**D**) Fluorescently labeled HEPM-EXO (red) were internalized by HIOEC. Bar= 20 μm.

HEPM-EXO Inhibited the Expansion but Facilitated the Migration of HIOEC

We evaluated the effects of HEPM-EXO on cell expansion, migration, apoptosis and epithelial-mesenchymal transition (EMT) of HOIEC by integrated methods. The viability and proliferation of HIOEC were determined by CCK8 and EdU incorporation assay, respectively. Compared with the control group, the cell viability of HIOEC beyond exosome stimulation declined significantly with the prolongation of culture time (Figure 3A). Similarly, the results of the EdU staining assay also showed a significantly reduction in proliferating cells in the HEPM-EXO treatment groups (Figure 3B and C). For cell migration detection, the scratch (Figure 4A and B) and Transwell (Figure 4C and D) assays were performed and the results both showed that HEPM-EXO significantly accelerated the migration of HIOEC. However, no significant difference was found among the exosome treatment groups and the PBS control group in EMT analyzed by Western blot (Figure 5A and B) and in cell apoptosis measured by TUNEL staining (Figure 5C and D). Taken together, these results suggested that HEPM-EXO contains key factors that inhibit cell expansion but promote cell migration of HIOEC.
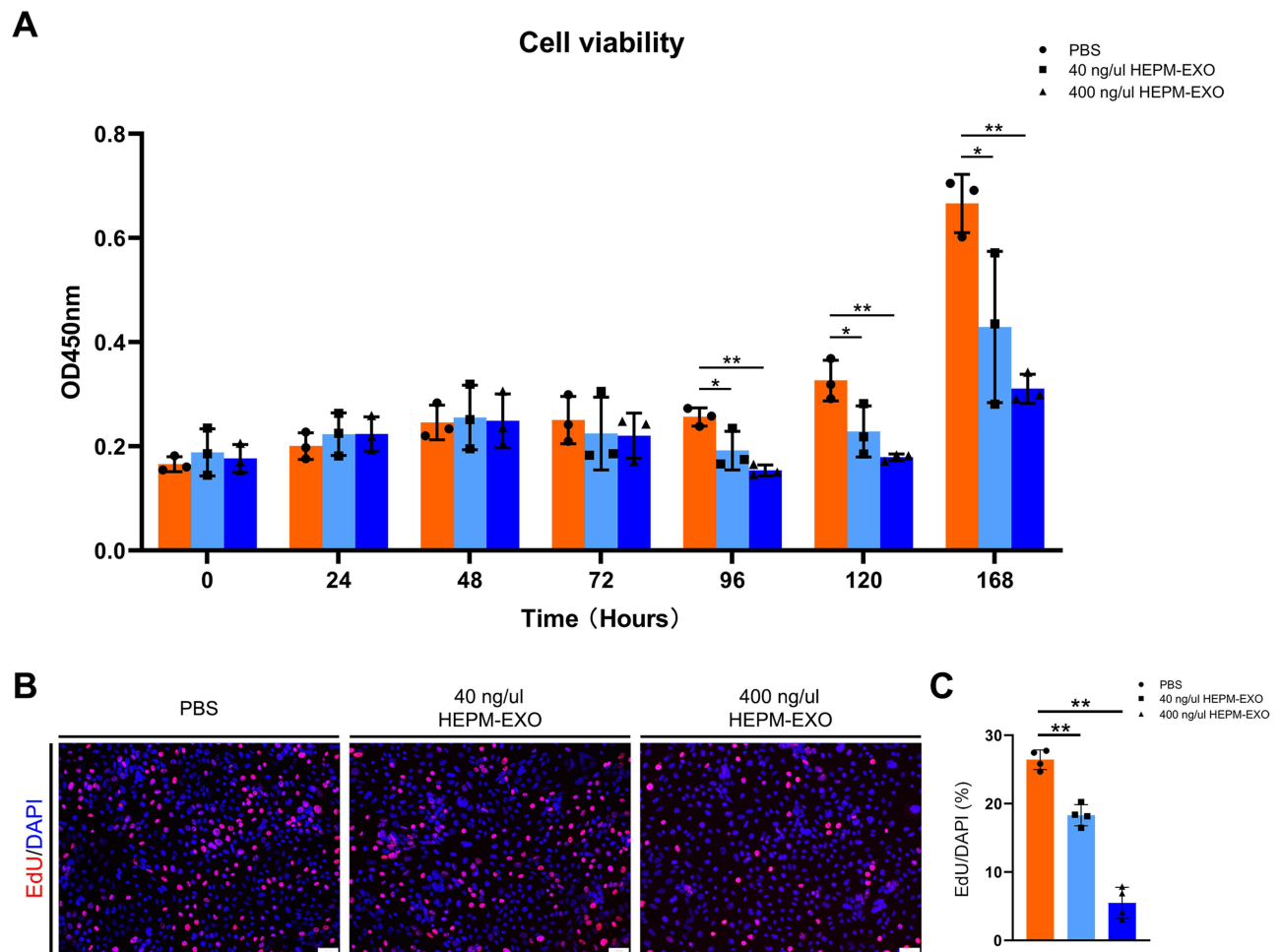
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A

B

C

Figure 3 Exposure of HEPM-EXO significantly decreased cell expansion of HIOEC. (**A**) Cell viability was assessed with cell counting kit-8 (CCK-8) assay. (**B**) Cell proliferation was detected by EdU incorporation. EdU staining (red) marked proliferating cells. Bar=50 μm. (**C**) Quantitative analysis of EdU incorporation assay. *$p < 0.05$. **$p < 0.01$.

Proteomic Analysis Revealed Possible Mechanism of HEPM-EXO on Cell Proliferation and Migration

To explore the potential mechanism by which HEPM-EXO regulated cell proliferation and migration, 4D label-free proteomic analysis of HEPM-EXO was conducted, in which the protein of HEPM cells was used as control for comparison. As shown in Figure 6A, 1183 and 5060 proteins were identified and quantified in HEPM-EXO and HEPM cells, respectively. 1055 proteins were found in both sets whereas 128 proteins were unique to the exosomes. In addition, 75 of the 1183 proteins in HEPM-EXO overlapped with the top 100 proteins that are often identified in exosomes listed in ExoCarta database (http://www.exocarta.org/),[14] confirming commonly found exosome proteins from other studies and ours. The representative exosome marker proteins with high expression level identified by mass spectrometry in HEPM-EXO were shown in Figure 6B, including PDCD6IP (ALIX), CD9, CD63, CD81, HSP90AA1, HSPA4, HSPA8, EGFR, ACTB and SDCBP.

Compared with HEPM cells, the expression level of 99 proteins were significantly up-regulated in HEPM-EXO, while 627 proteins were down-regulated (Figure 6C). A visual volcanic diagram of all proteins was shown in Figure 6D. Red dots represented highly expressed proteins in exosomes and green dots represented low expressed proteins. In HEPM cells, the most enriched proteins were ACTG1, ACTC1, GAPDH, TUBA1C and MT2A. However, the expression of these 5 proteins were significantly down-regulated in HEPM-EXO (Figure 6E). In contrast, the most significantly up-regulated proteins in HEPM-EXO compared with HEPM cells were POSTN, PXDN, SVEP1, FSTL1,
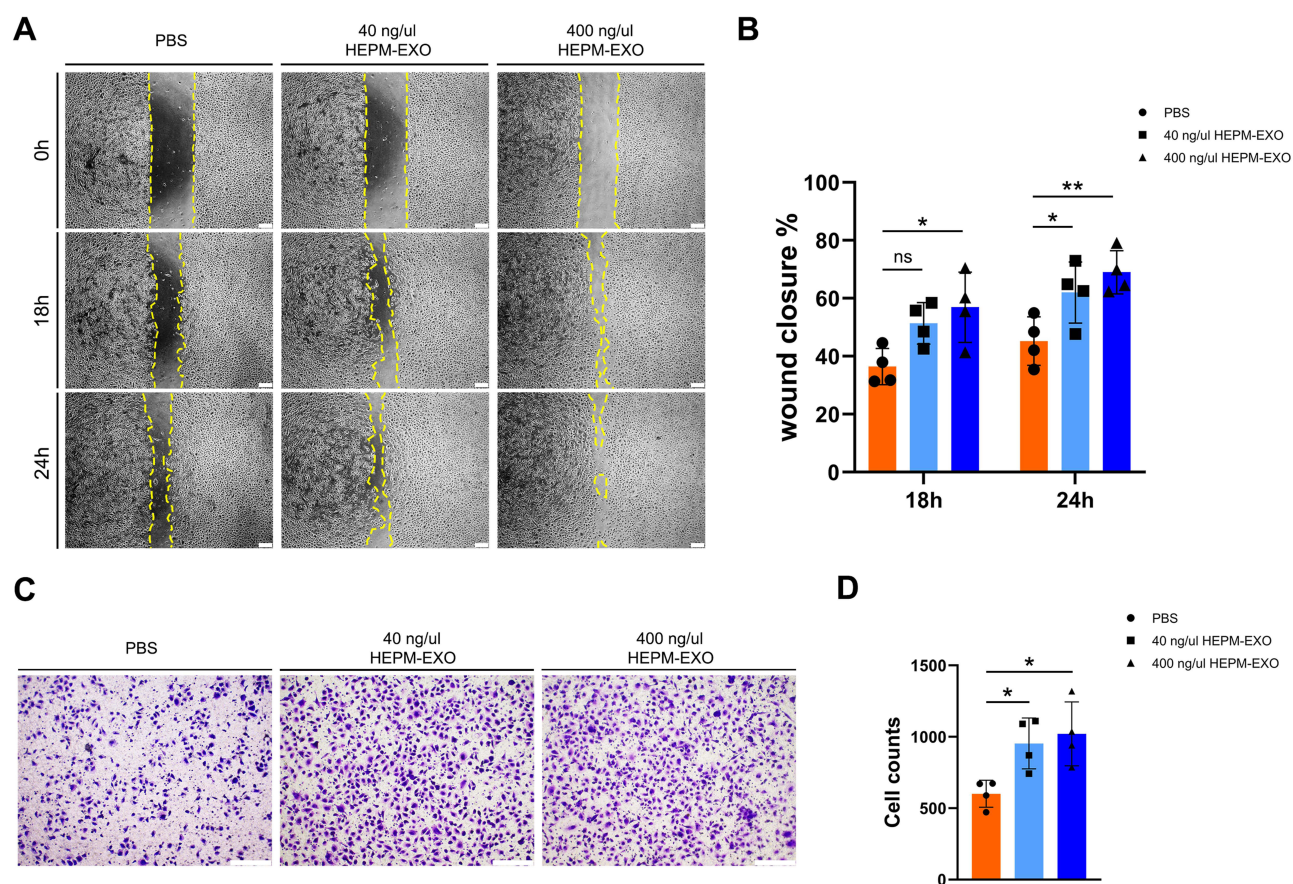
Figure 4 Exposure of HEPM-EXO significantly enhanced cell migration of HIOEC. **A**. Representative images of scratch assay. Bar=200 μm. **B**. Quantitative analysis of scratch assay showed that treatment of HEPM-EXO significantly accelerated wound closure of HIOEC. **C**. Representative images of Transwell assay. Bar=100 μm **D**. Quantitative analysis of Transwell assay showed that treatment of HEPM-EXO significantly facilitated migration of HIOEC. *p < 0.05. **p < 0.01.

THBS1, PTX3, NOTCH2, COL6A1, LOXL2 and IGF2R (Figure 6F). Protein-protein interaction (PPI) of these proteins were generated by STRING database and the result uncovered a highly interconnected network. Most proteins functioned in two or multiple pathways (Figure 6G). The expression levels of POSTN and THBS1 were further validated by Western blot. Equal amounts of protein were used in HEPM-EXO and HEPM group. Because the proteomic analysis showed that the expression of β-ACTIN was not significantly different between HEPM-EXO and HEPM cells (data not shown), *β-ACTIN* was used as housekeeping gene in the Western blot experiment. The results of Western blot (Figure 6H) were consistent with the proteomic analysis. The expression levels of POSTN and THBS1 were dramatically up-regulated in HEPM-EXO, while the expression level of β-ACTIN was not evidently different between HEPM-EXO and HEPM cells.

To detect the components and functions of proteins in HEPM-EXO, we performed gene ontology (GO) analysis of all the proteins contained in HEPM-EXO by using Blast2GO. As shown in Figure 6I, the cellular components of exosome proteins were largely assigned to extracellular space, extracellular region, collagen-containing extracellular matrix and cytosol, which were consistent with the characteristics of exosomes. The molecular functions were mainly involved in calcium ion binding, integrin binding, extracellular matrix structural constituent, structural constituent of cytoskeleton and so on (Figure 6J). The result of biological process analysis was shown in Figure 6K. We noticed that the prominent enriched biological processes were closely related to wound healing, including immune response (neutrophil degranulation and platelet degranulation), Wnt signaling pathway (planar cell polarity pathway), MAPK cascade, extracellular matrix (ECM) organization and neurogenesis (positive regulation of axon extension). These processes were highlighted in red boxes in the figure.
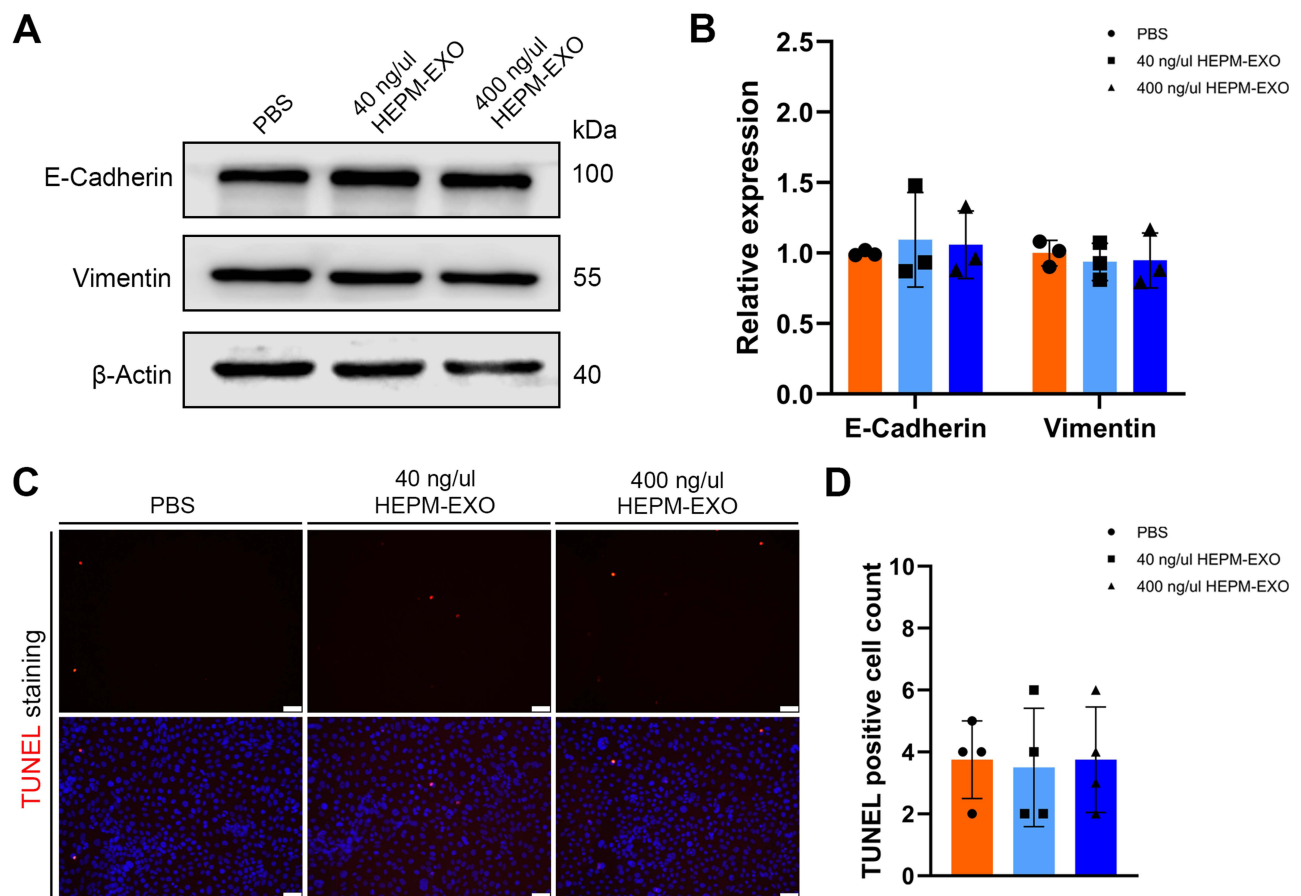
Figure 5 Exposure of HEPM-EXO exerted no significant effects on epithelial-mesenchymal transition (EMT) or apoptosis of HIOEC. (**A**) EMT was analyzed by Western blot. (**B**) Quantitative analysis of Western blot showed that no significant effects on EMT of HIOEC was observed upon HEPM-EXO stimulation. (**C**) Representative images of TUNEL staining. Bar=100 μm. (**D**) Quantitative analysis of TUNEL staining showed that treatment of HEPM-EXO did not influence the cell apoptosis of HIOEC.

Discussion

Acting as the "messengers" in cell-cell communications, exosomes are involved in many physical processes, including organogenesis.[16] For instance, during nephrogenesis, exposure of exosomes derived from embryonic kidney to meta-nephric mesenchyme cells resulted in advanced cellular organization.[17] In addition, treatment with human induced pluripotent stem cell (hiPSC) derived exosomes facilitated neurogenesis by enhancing neuronal cell proliferation and differentiation, suggesting that exosomes carry signaling components that regulate neural circuit development.[18] However, there are limited data on exosomes derived from embryonic palatal cells. Therefore, the putative role of palatal cell secreted exosomes in palatogenesis is still unknown. Here we show for the first time that exosomes present in palatal epithelial and mesenchymal cell from the very beginning of palatogenesis till palatal shelves meet. We were able to isolate exosomes from HEPM cells and co-culture them with HIOEC to investigate their potency. Our results showed that exosomes existed in the acellular base membrane in vivo, and the mesenchyme exosomes were capable of being incorporated by oral epithelial cells in vitro, indicating the possibility of exosomal mediated epithelial-mesenchymal crosstalk during palatal development.

The theories on mechanism underlying the degradation of MEE cells during palatal fusion are controversial. The predominant three theories are: (I) cell apoptosis; (II) cell migration; and (III) EMT.[19] The prevailing evidences suggested a vital role of cell apoptosis.[20,21] However, a very recent study combining static- and live- imaging approaches uncovered a particular form of collective migration of epithelial cells during palatal fusion, demonstrating that cell migration is crucial to this morphogenetic event.[22] Interestingly, our results disclosed that treatment of palatal
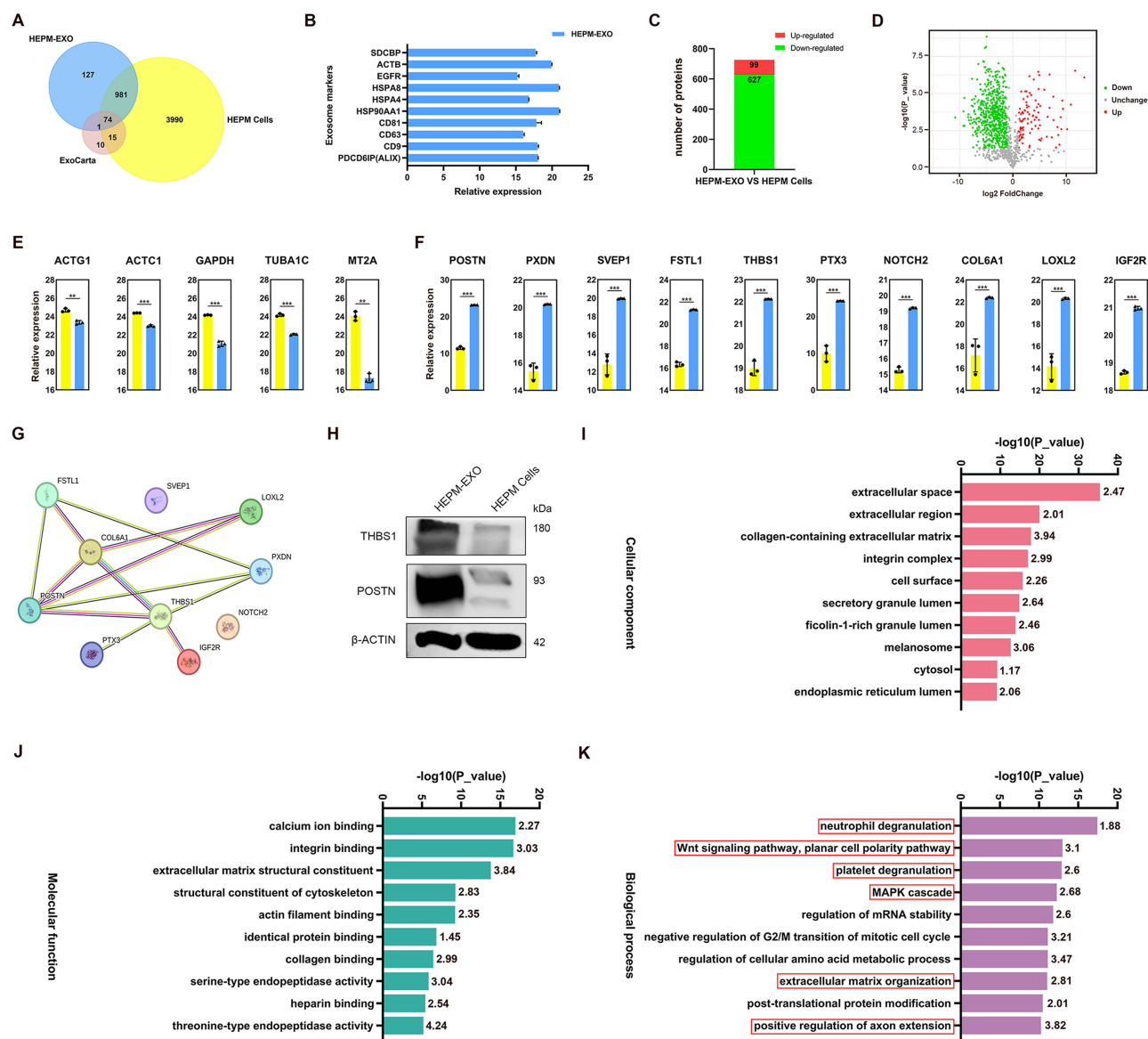
Figure 6 Bioinformatics analysis of proteins in HEPM-EXO. (**A**) Venn diagram showing the common and unique number of proteins in HEPM-EXO, HEPM cells and ExoCarta database. Venn diagrams were generated using FunRich.[15] (**B**) Relative expression levels of exosome markers in HEPM-EXO. (**C**) Numbers of differentially expressed proteins in HEPM-EXO compared with HEPM cells. (**D**) Volcano plot of proteins in HEPM-EXO and HEPM cells. (**E**) Top 5 enriched proteins in HEPM cells. (**F**) Top 10 significantly up-regulated proteins in HEPM-EXO compared with HEPM cells. (**G**) Protein-protein interactions among the top 10 proteins analyzed by STRING. (**H**) Expression level of THBS1, POSTN and β-ACTIN in HEPM-EXO and HEPM cells were detected by Western blot. (**I**) Cellular component analysis of proteins in HEPM-EXO. (**J**) Molecular function analysis of proteins in HEPM-EXO. (**K**) Biological process analysis of proteins in HEPM-EXO. The processes related to wound healing were highlighted in red boxes. The numbers above the bar represented the RichFactor value. **$*p < 0.01$. ***$p < 0.001$.

mesenchyme exosomes significantly facilitated epithelial cell migration. Thus, our study may provide a novel insight into the mechanism on the regulation of epithelial cell migration as well as cell-cell interactions during tissue fusion.

Over the past few years, exosomes have been studied mainly as biomarkers in the process of palatal physiology and pathology. Jia et al discovered that PIWI-interacting RNAs (piRNAs) in plasma exosomes from pregnant women acted as potential non-invasive prenatal biomarkers for the early diagnosis of nonsyndromic cleft lip and palate (nsCLP) before the defects could be screened using ultrasound inspection.[23] The team later identified let-7 cluster miRNAs in plasma exosomes as promising prenatal biomarkers for nsCLP.[24] However, our study was the first to uncover the influence of exosomes in cell-cell communication during palate development and may provide new evidence for the involvement of exosomes in organogenesis.

In this study, we analyzed the proteins encapsulated in HEPM-EXO and HEPM cells by 4D label-free quantitative proteomics to reveal the overall and distinctive functions of HEPM-EXO. Our results exhibited massive differences between exosomal and cellular proteins, which may be the reason why HEPM-EXO and HEPM cells functioned dissimilarly. The top 10 proteins which were significantly up-regulated in HEPM-EXO showed a high intercorrelation and were reported to be associated with cell growth, cell communication and signal transduction.[25–34] HEPM-EXO may rely on some of these proteins to regulate the proliferation and migration of HIOEC. Among these proteins, THBS1 was frequently associated with exosomal regulation of cell migration. Xiao et al showed that oral squamous cell carcinoma (OSCC) cells-derived exosomal THBS1 participated in macrophage polarization to an M1-like phenotype through p38, Akt, and SAPK/JNK signaling, ultimately fostering the migration of OSCC cells.[35] Likewise, Patwardhan et al showed that THBS1 in extracellular matrix (ECM) stiffness-tuned exosomes was identified as a pivotal regulator that provokes ECM stiffness-dependent breast cancer motility.[36] However, Liao et al showed that canine endothelial colony-forming cells (ECFC)-derived exosomal THBS1 negatively regulated the migration of Human umbilical vein endothelial cells (HUVECs) via the PI3K/AKT/ERK pathway.[37] Altogether, exosomal THBS1 may regulate cell migration in a cell/tissue context-specific manner. However, in our study, whether HEPM-EXO fostered epithelial cell migration through THBS1 regulation requires further verification.

In the results of our GO analysis of HEPM-EXO, some of the most enriched biological processes were indirectly involved in cell proliferation and migration. Besides, we surprisingly observed that the prominent enriched biological processes were closely associated with wound healing, which is a highly sequential process involving a series of temporally overlapping events, including hemostasis, inflammation, proliferation, and tissue remodeling.[38] At present, multiple clinically conventional approaches, such as negative pressure suction and autologous skin grafts, are applied to facilitate wound healing, but all have certain benefits and drawbacks.[39–41] Recently, exosomes derived from mesenchymal stem cells (MSCs) are increasingly discovered to be potent to accelerate wound healing. Compared to MSCs, MSC-derived exosomes possess lower immunogenicity, easy storage, and highly effective biological activity.[42] Considering that in our study, HEPM-EXO substantially enhanced cell migration and contained proteins related to multiple wound healing processes, we assumed that HEPM-EXO might be potential to promote wound healing. However, the biological superiority of HEPM-EXO over other MSC-derived exosomes needs to be further evaluated in detail.

Conclusion

In this study, we show for the first time the presence of exosomes in palatal epithelial and mesenchymal cells during embryonic palate development. Our results give a first insight into the role of HEPM-EXO for cell-cell communication during palatal fusion and provide new evidence for the involvement of exosomes in organogenesis. These exosomes carry proteins that are critical for wound healing processes. Hopefully with further research, HEPM-EXO may be a potential therapeutic opportunity for wound healing.

Data Sharing Statement

Data used and analyzed during the current study are available from the corresponding author on reasonable request.

Consent for Publication

All the authors consent for publication.

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Disclosure

The authors report no conflicts of interest in this work.

References

1. Won HJ, Kim JW, Won HS, Shin JO. Gene regulatory networks and signaling pathways in palatogenesis and cleft palate: a comprehensive review. *Cells*. 2023;12(15). doi:10.3390/cells12151954
2. Hammond NL, Dixon MJ. Revisiting the embryogenesis of lip and palate development. *Oral Dis*. 2022;28(5):1306–1326. doi:10.1111/odi.14174
3. Ray HJ, Niswander L. Mechanisms of tissue fusion during development. *Development*. 2012;139(10):1701–1711. doi:10.1242/dev.068338
4. Iwaya C, Suzuki A, Iwata J. MicroRNAs and gene regulatory networks related to cleft lip and palate. *Int J Mol Sci*. 2023;24:4.
5. Lan Y, Jiang R. Sonic hedgehog signaling regulates reciprocal epithelial-mesenchymal interactions controlling palatal outgrowth. *Development*. 2009;136(8):1387–1396. doi:10.1242/dev.028167
6. Smith TM, Lozanoff S, Iyyanar PP, Nazarali AJ. Molecular signaling along the anterior-posterior axis of early palate development. *Front Physiol*. 2012;3:488. doi:10.3389/fphys.2012.00488
7. Van Niel G, Carter DRF, Clayton A, Lambert DW, Raposo G, Vader P. Challenges and directions in studying cell-cell communication by extracellular vesicles. *Nat Rev Mol Cell Biol*. 2022;23(5):369–382. doi:10.1038/s41580-022-00460-3
8. Kalluri R, LeBleu VS. The biology, function, and biomedical applications of exosomes. *Science*. 2020;367:6478. doi:10.1126/science.aau6977
9. Mathieu M, Martin-Jaular L, Lavieu G, Théry C. Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. *Nat Cell Biol*. 2019;21(1):9–17. doi:10.1038/s41556-018-0250-9
10. Jiang N, Xiang L, He L, et al. Exosomes mediate epithelium-mesenchyme crosstalk in organ development. *ACS Nano*. 2017;11(8):7736–7746. doi:10.1021/acsnano.7b01087
11. Hayashi T, Lombaert IM, Hauser BR, Patel VN, Hoffman MP. Exosomal MicroRNA transport from salivary mesenchyme regulates epithelial progenitor expansion during organogenesis. *Dev Cell*. 2017;40(1):95–103. doi:10.1016/j.devcel.2016.12.001
12. Yue C, Cao J, Wong A, et al. Human bone marrow stromal cell exosomes ameliorate periodontitis. *J Dent Res*. 2022;101(9):1110–1118. doi:10.1177/00220345221084975
13. Khan NZ, Cao T, He J, et al. Spinal cord injury alters microRNA and CD81+ exosome levels in plasma extracellular nanoparticles with neuroinflammatory potential. *Brain Behav Immun*. 2021;92:165–183. doi:10.1016/j.bbi.2020.12.007
14. Keerthikumar S, Chisanga D, Ariyaratne D, et al. ExoCarta: a web-based compendium of exosomal cargo. *J Mol Biol*. 2016;428(4):688–692. doi:10.1016/j.jmb.2015.09.019
15. Fonseka P, Pathan M, Chitti SV, Kang T, Mathivanan S. FunRich enables enrichment analysis of OMICs datasets. *J Mol Biol*. 2021;433 (11):166747. doi:10.1016/j.jmb.2020.166747
16. Gurung S, Perocheau D, Touramanidou L, Baruteau J. The exosome journey: from biogenesis to uptake and intracellular signalling. *Cell Commun Signal*. 2021;19(1):47. doi:10.1186/s12964-021-00730-1
17. Krause M, Rak-Raszewska A, Naillat F, et al. Exosomes as secondary inductive signals involved in kidney organogenesis. *J Extracell Vesicles*. 2018;7(1):1422675. doi:10.1080/20013078.2017.1422675
18. Sharma P, Mesci P, Carromeu C, et al. Exosomes regulate neurogenesis and circuit assembly. *Proc Natl Acad Sci U S A*. 2019;116 (32):16086–16094. doi:10.1073/pnas.1902513116
19. Nakajima A, Gulka AOD, Hanai JI. TGF-β signaling and the epithelial-mesenchymal transition during palatal fusion. *Int J Mol Sci*. 2018;19:11.
20. Cuervo R, Covarrubias L. Death is the major fate of medial edge epithelial cells and the cause of basal lamina degradation during palatogenesis. *Development*. 2004;131(1):15–24. doi:10.1242/dev.00907
21. Iseki S. Disintegration of the medial epithelial seam: is cell death important in palatogenesis? *Dev Growth Differ*. 2011;53(2):259–268. doi:10.1111/j.1440-169X.2010.01245.x
22. Teng T, Teng CS, Kaartinen V, Bush JO. A unique form of collective epithelial migration is crucial for tissue fusion in the secondary palate and can overcome loss of epithelial apoptosis. *Development*. 2022;2:149.
23. Jia S, Zhang Q, Wang Y, et al. PIWI-interacting RNA sequencing profiles in maternal plasma-derived exosomes reveal novel non-invasive prenatal biomarkers for the early diagnosis of nonsyndromic cleft lip and palate. *EBioMedicine*. 2021;65:103253. doi:10.1016/j.ebiom.2021.103253
24. Jia S, Zhang Q, Wang Y, et al. Identification by RNA-Seq of let-7 clusters as prenatal biomarkers for nonsyndromic cleft lip with palate. *Ann N Y Acad Sci*. 2022;1516(1):234–246. doi:10.1111/nyas.14868
25. Torrente Y, Bella P, Tripodi L, Villa C, Farini A. Role of insulin-like growth factor receptor 2 across muscle homeostasis: implications for treating muscular dystrophy. *Cells*. 2020;9:2.
26. Wen B, Xu LY, Li EM. LOXL2 in cancer: regulation, downstream effectors and novel roles. *Biochim Biophys Acta Rev Cancer*. 2020;1874 (2):188435. doi:10.1016/j.bbcan.2020.188435
27. Zhang Y, Liu Z, Yang X, et al. H3K27 acetylation activated-COL6A1 promotes osteosarcoma lung metastasis by repressing STAT1 and activating pulmonary cancer-associated fibroblasts. *Theranostics*. 2021;11(3):1473–1492. doi:10.7150/thno.51245
28. Mesini N, Fiorcari S, Atene CG, et al. Role of Notch2 pathway in mature B cell malignancies. *Front Oncol*. 2022;12:1073672. doi:10.3389/fonc.2022.1073672
29. Massimino AM, Colella FE, Bottazzi B, Inforzato A. Structural insights into the biological functions of the long pentraxin PTX3. *Front Immunol*. 2023;14:1274634. doi:10.3389/fimmu.2023.1274634
30. Kaur S, Roberts DD. Why do humans need thrombospondin-1? *J Cell Commun Signal*. 2023;17(3):485–493. doi:10.1007/s12079-023-00722-5
31. Gu C, Xue H, Yang X, Nie Y, Qian X. Role of follistatin-like protein 1 in liver diseases. *Exp Biol Med*. 2023;248(3):193–200. doi:10.1177/15353702221142604
32. Elenbaas JS, Jung IH, Coler-Reilly A, Lee PC, Alisio A, Stitziel NO. The emerging Janus face of SVEP1 in development and disease. *Trends Mol Med*. 2023;29(11):939–950. doi:10.1016/j.molmed.2023.08.002
33. Cheng G, Shi R. Mammalian peroxidasin (PXDN): from physiology to pathology. *Free Radic Biol Med*. 2022;182:100–107. doi:10.1016/j.freeradbiomed.2022.02.026

34. Balbi C, Milano G, Fertig TE, et al. An exosomal-carried short periostin isoform induces cardiomyocyte proliferation. *Theranostics*. 2021;11 (12):5634–5649. doi:10.7150/thno.57243

35. Xiao M, Zhang J, Chen W, Chen W. M1-like tumor-associated macrophages activated by exosome-transferred THBS1 promote malignant migration in oral squamous cell carcinoma. *J Exp Clin Cancer Res*. 2018;37(1):143. doi:10.1186/s13046-018-0815-2

36. Patwardhan S, Mahadik P, Shetty O, Sen S. ECM stiffness-tuned exosomes drive breast cancer motility through thrombospondin-1. *Biomaterials*. 2021;279:121185. doi:10.1016/j.biomaterials.2021.121185

37. Liao F, Liao Z, Zhang T, et al. ECFC-derived exosomal THBS1 mediates angiogenesis and osteogenesis in distraction osteogenesis via the PI3K/ AKT/ERK pathway. *J Orthop Translat*. 2022;37:12–22. doi:10.1016/j.jot.2022.08.004

38. Wang PH, Huang BS, Horng HC, Yeh CC, Chen YJ. Wound healing. *J Chin Med Assoc*. 2018;81(2):94–101. doi:10.1016/j.jcma.2017.11.002

39. Veith AP, Henderson K, Spencer A, Sligar AD, Baker AB. Therapeutic strategies for enhancing angiogenesis in wound healing. *Adv Drug Deliv Rev*. 2019;146:97–125. doi:10.1016/j.addr.2018.09.010

40. Luo R, Dai J, Zhang J, Li Z. Accelerated skin wound healing by electrical stimulation. *Adv Healthc Mater*. 2021;10(16):e2100557. doi:10.1002/ adhm.202100557

41. Freedman BR, Hwang C, Talbot S, Hibler B, Matoori S, Mooney DJ. Breakthrough treatments for accelerated wound healing. *Sci Adv*. 2023;9(20): eade7007. doi:10.1126/sciadv.ade7007

42. Zhou C, Zhang B, Yang Y, et al. Stem cell-derived exosomes: emerging therapeutic opportunities for wound healing. *Stem Cell Res Ther*. 2023;14 (1):107. doi:10.1186/s13287-023-03345-0